Contrasting pH-Optima of Light-Driven O_2- and H_2O_2-Reduction in Spinach Chloroplasts as Measured *via* Chlorophyll Fluorescence Quenching

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Quenching analysis of chlorophyll fluorescence by the saturation pulse method is used to investigate the pH-dependency of O_2-dependent electron flow in intact spinach chloroplasts with high ascorbate peroxidase activity. When carboxylase/oxygenase activity is blocked, photochemical and non-photochemical quenching are initially low and increase with illumination time. Quenching shows a pH-optimum around pH 6.5, but only when ΔpH-formation is allowed. It is suggested that overall O_2-dependent electron flow involves two major components, namely O_2-reduction (Mehler reaction) and reduction of the H_2O_2 formed in the Mehler reaction, involving enzymic activity of ascorbate peroxidase and monodehydroascorbate reductase. The separated pH-dependencies of light driven O_2-reduction (presence of KCN) and of H_2O_2-reduction (anaerobic conditions) reveal contrasting pH-optima around pH 5 and 8.5, respectively. Energy-dependent, dark relaxable non-photochemical quenching is not observed with O_2-reduction but with H_2O_2-reduction, and only at pH-values above 6.5. The relevance of these findings with respect to regulation of photosynthetic electron flow is discussed. It is suggested that upon limitation of assimilatory electron flow O_2-dependent non-assimilatory flow is responsible for ΔpH-formation, by which it is autocatalytically stimulated. It is proposed that this autocatalytical reaction sequence is the basis of the so-called "Kautsky effect" of chlorophyll fluorescence induction.

Introduction

Recent progress in instrumentation and methodology [1–2] has rendered chlorophyll fluorescence a reliable indicator of photosynthetic electron transport in intact leaves [3–7] and chloroplasts [8–12]. Quenching analysis by the so-called "saturation pulse method", which is based on the original "light-doubling method" of Bradbury and Baker [13], allows to separate photochemical and non-photochemical components of overall quenching [1, 14–16]. Photochemical quenching, which is suppressed by a pulse of saturating light, reflects the relative rate of charge separation at PS II reaction centers. For the actual electron transport rate not only the "openness" of PS II centers but also their intrinsic photochemical efficiency is essential, which is reflected by F_V/F_M [3, 6, 17, 18], with F_V corresponding to the increase of fluorescence yield when all centers are transformed from the open to the closed state, and F_M representing the maximal fluorescence yield with all centers closed, *e.g.* by application of a saturation pulse. Non-photochemical quenching is closely correlated with the lowering of F_V/F_M. A major component of non-photochemical quenching is caused by "membrane energization" [19] which depends on the formation of a transthylakoidal pH-gradient (ΔpH) and, hence, is called "energy-dependent quenching". As membrane energization leads to a lowering of F_V/F_M, it results in "down-regulation" of PS II, essentially by increasing the rate constant of thermal energy dissipation with respect to that of photochemistry. The mechanism of non-photochemical quenching is not yet fully understood. Major problems are the existence of several forms of non-photochemical quenching [16, 20–22] and the difficulty in distinguishing between cause and action [23, 24].

Down-regulation of PS II, as reflected by a decrease of F_V/F_M, is of utmost importance for the protection of the photosynthetic apparatus against photoinhibitory damage [25, 26]. There is general agreement that the ΔpH is an intermediate in this process. Recently, we have drawn attention to the role of O_2-dependent electron transport in the creation of the ΔpH [8, 10, 12, 24]. With the given stoichiometric demands of ATP/NADPH of the

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Calvin cycle, any surplus ΔpH should depend on non-assimilatory electron flow, as *e.g.* on O_2-reduction or cyclic flow. So far, the evidence for substantial PS I or PS II cyclic flow in the presence of O_2 is not convincing (see *e.g.* ref. [24, 27, 28]). On the other hand, O_2-reduction has been well documented by a large number of studies [29–36].

There is another type of non-assimilatory electron flow, namely the reduction of internally formed H_2O_2, which has been characterized as an efficient detoxification mechanism [37–41], but so far has not found much attention regarding its possible regulatory role. In recent reports, we have shown that H_2O_2-reduction is reflected by pronounced fluorescence quenching [10, 12, 24]. When H_2O_2 is formed internally by O_2-reduction, the combination of the formation and detoxification reactions does not lead to net O_2-exchange (see reviews in ref. [42 and 43]), making a detection by standard gas exchange methods impossible:

$$2H_2O \rightarrow 4e^- + 4H^+ + O_2 \quad (1)$$

$$4O_2 + 4e^- \rightarrow 4\dot{O}_2^- \quad (2)$$

$$4\dot{O}_2^- + 4H^+ \rightarrow 2O_2 + 2H_2O_2 \quad (3)$$

$$2H_2O \rightarrow O_2 + 4H^+ + 4e^- \quad (4)$$

$$2H_2O_2 + 4e^- + 4H^+ \rightarrow 4H_2O \quad (5)$$

Reactions (1)–(2) are associated with O_2-reduction (Mehler reaction), reaction (3) represents the enzymic dismutation of the initially formed superoxide radical anions to O_2 and H_2O_2, and reactions (4)–(5) represent the enzymic reduction of H_2O_2 by electrons derived from water splitting. With most chloroplast preparations used in previous studies, reactions (4)–(5) were not active because no care was taken to preserve the activity of the ascorbate peroxidase. For this enzyme to be active, an exceptionally high degree of chloroplast intactness and presence of ascorbate are essential [41, 44]. When catalase is added, as in many previous studies, internal H_2O_2-reduction *via* the ascorbate peroxidase is partially suppressed. In this case, net O_2-exchange is also zero, but chlorophyll fluorescence measurements clearly indicate that an important part of overall electron flow is suppressed when catalase is added, as photochemical and energy-dependent quenching are decreased (see *e.g.* Fig. 1 of ref. [10]).

Previous work has shown that there is a close link between O_2-dependent electron flow and ΔpH-formation, as expressed in energy-dependent fluorescence quenching (see *e.g.* Figs. 1–5 of ref. [24]). In the present report, the pH-dependencies of overall O_2-dependent electron flow, as well as of the separated partial reactions of O_2-reduction and H_2O_2-reduction are investigated. Contrasting pH-optima for these partial reactions are found and data are presented which suggest an autocatalytic activation of O_2-dependent electron flow by ΔpH-formation.

Materials and Methods

Intact chloroplasts were isolated from freshly harvested leaves of greenhouse-grown spinach following the method described by Asada *et al.* [28], which involves a purification step by centrifugation through a layer of 40% Percoll (v/v). The resulting chloroplasts were 90–98% intact, as determined by the ferricyanide method [45].

Chloroplasts were suspended isotonically at a final concentration of 50 μg Chl·ml^{-1} in reaction media containing 330 mM sorbitol, 1 mM $MgCl_2$, 0.25 mM Na_2PO_4, 10 mM Na-ascorbate and 50 mM K-Tricine (for pH values between 8 and 9) or 50 mM K-Hepes (for pH values between 6 and 7.5) or 50 mM K-MES (for pH values between 5 and 6) or 50 mM K-MES-glycine (for pH values between 4 and 5). Chloroplasts were kept in these media for 10 min before the start of measurements, to allow equilibration of the external pH with the chloroplast interior. Solutions of 0.1 M H_2O_2 were freshly prepared from 30% (v/v) H_2O_2 (Perhydrol, Merck) and not used for longer than 3 h in a series of experiments. Temperature was 20 °C or 12 °C, as indicated in the figure legends.

Modulated chlorophyll fluorescence was measured with a PAM Chlorophyll Fluorometer (Walz, Effeltrich, Germany) as previously described [1]. Actinic red light was obtained from a halogen lamp (Xenophot, Osram) equipped with a cut-off filter (Schott RG 645) and various neutral density filters (Schott NG series). Saturating pulses of white light were applied with a commercial pulse lamp (FL 103, Walz) equipped with a short-pass filter (DT Cyan, $\lambda < 700$ nm, Balzers).

Results and Discussion

In Fig. 1 light-induced and H_2O_2-induced fluorescence changes of intact spinach chloroplasts at

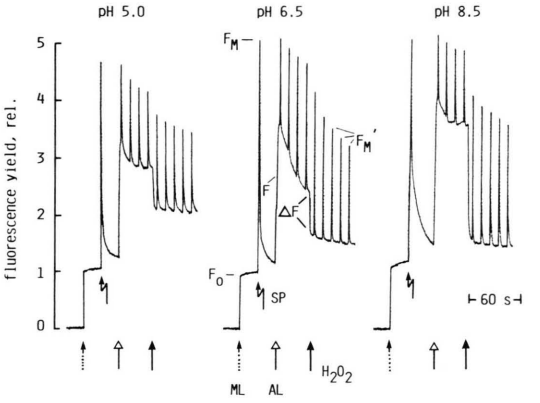

Fig. 1. Light- and H_2O_2-induced fluorescence changes in intact spinach chloroplasts suspended isotonically at different pH-values. Presence of 10 mM glycerinaldehyde. Actinic light intensity, 20 W/m^2; temperature 20 °C. Final concentration of H_2O_2, 0.2 mM. The points in time at which the various light sources are switched on and H_2O_2 is injected are indicated by arrows: ML, measuring light; AL, actinic light; SP, saturation pulse. Notations for fluorescence yields are: F_o, minimal fluorescence of dark-adapted sample; F_M, maximal fluorescence of dark-adapted sample; F_M', maximal fluorescence of illuminated sample induced by saturation pulses; ΔF, rapid change in fluorescence yield (F) induced by H_2O_2. For other conditions, see Materials and Methods.

different pH-values are compared. Chloroplasts with a very high degree of intactness were used (see Materials and Methods) which in the presence of ascorbate display high activity of ascorbate peroxidase. In this experiment 10 mM glycerinaldehyde was present, to prevent carboxylation/oxygenation reactions [46]. However, very similar results were also obtained in the absence of glycerinaldehyde. The measuring procedure shall be detailed for the pH 6.5 curve of Fig. 1: When a weak modulated measuring light (ML) is switched on the minimal fluorescence yield, F_o, is registered. Upon application of a saturating light pulse (SP) the maximal fluorescence yield of the dark-adapted sample, F_M, is measured. With the application of continuous actinic light (AL) fluorescence yield, F, rapidly rises to a peak, which is below F_M, from where it slowly decays towards a steady state level. In the peak and then every 10 s saturating light pulses are applied to register the maximal fluorescence yield during continuous illumination, F_M'. Before reaching the steady state, a small volume of H_2O_2 is injected into the rapidly stirred chloroplast suspension to a final concentration of 0.2 mM. H_2O_2 leads to a rapid lowering of fluorescence yield (ΔF), and to an enhancement of the F_M'-decline. Comparing the kinetic traces at the three different pH-values, it is apparent that the light-induced F-decline is most pronounced at pH 6.5 while the H_2O_2-induced F-decrease is largest at pH 8.5. For a quantitative comparison, the corresponding photochemical quenching coefficients, q_P, can be calculated [1, 47]. The q_P in continuous light is given by $(F_M'-F)/(F_M'-F_o)$ and the H_2O_2-induced photochemical quenching, $(\Delta q_P)H_2O_2$, corresponds to the difference between the q_P-values measured shortly after and before H_2O_2-addition. In Fig. 2 the pH-dependency of q_P (before H_2O_2-addition) and of $(\Delta q_P)H_2O_2$ is presented. The q_P displays a broad maximum around pH 6.5, whereas the $(\Delta q_P)H_2O_2$ is peaking around pH 8.5 with a possible minor peak around pH 5.5.

For an evaluation of the data in Figs. 1 and 2, it should be considered that these experiments were carried out with intact chloroplasts and that, hence, the internal pH of the chloroplasts may be shifted with respect to the external pH of the medium. This is particularly true for illuminated chloroplasts in which a transthylakoidal proton gradient (ΔpH) is built-up, resulting in lumen-acidification and stroma-alkalization. Light-induced ΔpH-formation could be expressed in the slow

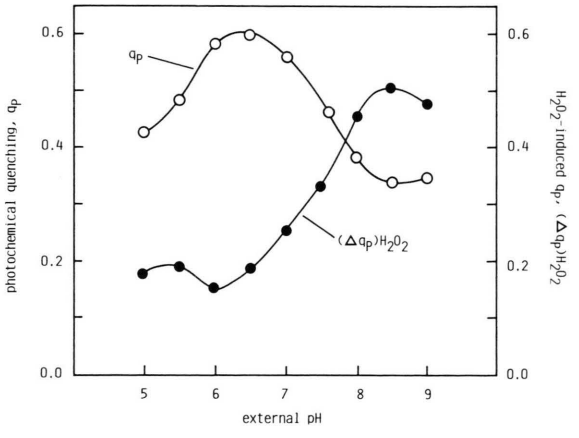

Fig. 2. Dependency of photochemical quenching, q_P, and H_2O_2-induced photochemical quenching, $(\Delta q_P)H_2O_2$, in illuminated intact chloroplasts on pH of external medium. Chloroplasts were illuminated for 40 s before q_P was evaluated and shortly afterwards H_2O_2 was injected to induce $(\Delta q_P)H_2O_2$. Conditions as for Fig. 1.

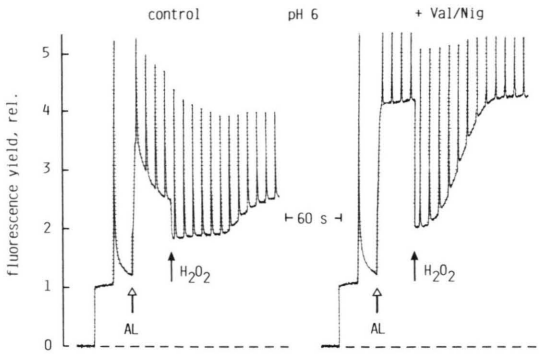

Fig. 3. Light- and H_2O_2-induced fluorescence changes at pH 6 in the absence and presence of valinomycin/nigericin. Conditions as for Fig. 1 and 2.

declines of F and F_M'. This assumption is substantiated by the results of Fig. 3. There the pH 6 curves are compared for absence and presence of valinomycin/nigericin (Val/Nig), which effectively suppresses ΔpH-formation. In the absence of the uncoupler there is a substantial increase of q_P from 0.39 to 0.60 within the first 40 sec of illumination, whereas in the presence of Val/Nig q_P remains at its initial value of 0.27.

Under the given experimental conditions, oxygen should be the major electron acceptor, and O_2-dependent electron flow should be primarily responsible for the observed photochemical and non-photochemical quenching. Furthermore, the results of Fig. 3 suggest that somehow O_2-dependent electron flow is enhanced by ΔpH-formation. The strong quenching induced by externally added H_2O_2 indicates that also when the H_2O_2 is formed internally, its reduction will contribute to overall O_2-dependent electron flow. The data of Fig. 2 appear to suggest that H_2O_2-reduction is favored by alkaline pH. If correct, this suggestion could present a reasonable explanation of the stimulation of O_2-dependent electron flow by ΔpH-formation, as apparent from Fig. 3. However, the data of Fig. 3 could also provide another interpretation of the pH-dependency of $(\Delta q_P)H_2O_2$ in Fig. 2. Obviously, there is sufficient activity of the H_2O_2-reducing system also at pH 6 to give a substantial increase of q_P upon H_2O_2-addition. It could be argued, that the increase of H_2O_2-induced quenching is just the consequence of complementarity between the q_P reached before H_2O_2-addition and the $(\Delta q_P)H_2O_2$. Of course, when q_P approaches unity before H_2O_2-addition, further stimulation by H_2O_2 would be prevented.

These considerations show that it is necessary to determine separately the pH-dependencies of the relevant partial reactions of overall O_2-dependent electron flow. The likely sequence of events, as it has been elucidated by extensive previous research (see *e.g.* reviews in ref. [42, 43]) may be summarized as follows: O_2-reduction → superoxide formation → H_2O_2-formation (catalyzed by superoxide dismutase) → mono-dehydroascorbate (MDA)-formation (catalyzed by ascorbate peroxidase) → oxidation of NADPH (catalyzed by MDA-reductase) → stimulation of NADP-dependent electron flow.

It is the reduction of O_2 and of H_2O_2 (*i.e. via* the enzymic steps resulting in regeneration of oxidized NADP) which produce the photochemical quenching, the pH-dependency of which is studied here. To our knowledge, not much information is available on the pH-dependency of O_2-reduction. According to Asada and co-workers [42] the superoxide anion is photoproduced in the aprotic interior of the thylakoid membrane in which the disproportionation of superoxide to H_2O_2 and O_2 is limited due to lack of protons, and the permeation of superoxide anion radicals through the thylakoid membrane is very slow. As protons are indispensable for the disproportionation of superoxide anion radicals, an enhancement of H_2O_2-formation by "membrane acidification" may be expected. As to the pH-optimum of the ascorbate peroxidase, different values are reported in the literature: Nakano and Asada [48] found an optimum at pH 7.0 for the ascorbate peroxidase in the stroma fraction of spinach chloroplasts, whereas Jablonski and Anderson [40] reported a pH-optimum at 8.2 for pea chloroplasts. The MDA-reductase was shown to display a broad pH-optimum at 6.8–9.0 [49].

In the context of the present investigation, it may be sufficient to separate the pH-dependencies of O_2-reduction on one hand and of H_2O_2-reduction, including ascorbate peroxidase and MDA-reductase activity, on the other hand. In principle, it should be possible to specifically study H_2O_2-reduction with chloroplasts in presence of the glucose/glucose oxidase system, which effectively removes O_2, and to specifically study O_2-reduc-

tion in the presence of KCN, which is known to inhibit ascorbate peroxidase [42]. These aspects are illustrated in Fig. 4 for an experiment at pH 8. Chloroplasts were suspended in a medium containing glucose and bubbled with nitrogen. Glucose oxidase was added briefly before onset of actinic illumination. When it was added earlier, dark inactivation of the ascorbate peroxidase occurred [41, 44], due to the H_2O_2 formed by divalent reduction of some O_2 still present in the suspension despite of N_2-bubbling [12, 42]. The effect of this H_2O_2 on fluorescence quenching can be seen upon onset of actinic illumination. There is a rapid fluorescence decline involving increases of photochemical and non-photochemical quenching, which is then reversed again, as the H_2O_2 becomes exhausted. A state is reached in which q_P is almost completely suppressed. The saturation pulses cause negative spikes, reflecting transient reduction of pheophytin, which is a fluorescence quencher [50]. Obviously, in this system O_2-reduction is eliminated and, hence, H_2O_2-reduction can be selectively studied. When H_2O_2 is added, a biphasic fluorescence decline is induced. The rapid phase is likely to reflect the spontaneous H_2O_2-induced increase of photochemical quenching, while the slower phase is mainly caused by an increase in non-photochemical quenching. This non-photochemical quenching can be relaxed by addition of nigericin (not shown in the figure) and, hence, represents energy-dependent quenching. When H_2O_2 becomes exhausted, first q_P and then q_N are suppressed again. The H_2O_2-effect can be repeated, and while H_2O_2 is still present as a substrate of the peroxidase, this can be inhibited by injection of KCN. It is apparent, that there is an immediate suppression of q_P and a slower relaxation of q_N. Another addition of H_2O_2 now does not produce fluorescence quenching anymore. Rather there is a small increase of fluorescence yield, which may result from a trace of O_2 injected with the H_2O_2, which would cause relaxation of pheophytin quenching. These results suggest that the conditions stated above for selective study of H_2O_2- and O_2-reduction are suitable.

In Fig. 5 original traces for determination of H_2O_2-reduction at pH 6.0 and 8.5 are presented. Samples were pre-treated as in Fig. 4. It is apparent that H_2O_2-induced quenching is substantially larger at pH 8.5 than at pH 6.0. Furthermore, the non-photochemical quenching induced at pH 8.5 is fully reversible upon darkening, while it is irreversible at pH 6.0. Therefore, although obviously there is some peroxidase activity at pH 6.0, this appears to be relatively low and, most importantly, it does not produce the "membrane energization", which is supposed to play a decisive regulatory role in photosynthesis. In Fig. 6 the full pH-dependencies of H_2O_2-induced photochemical and energy-dependent (*i.e.* relaxable) quenching are shown. As in Fig. 2, the main pH-optimum is

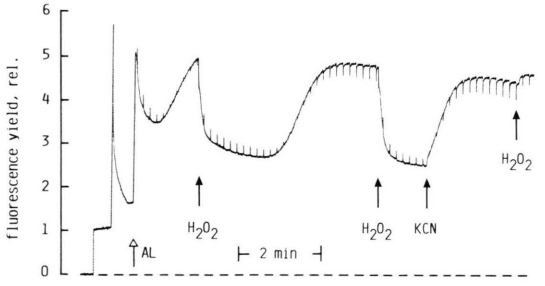

Fig. 4. Properties of H_2O_2-induced fluorescence quenching in the absence of molecular oxygen. Chloroplast suspension at pH 8; presence of 10 mM glucose. The suspension was bubbled for 5 min with N_2-gas before closure of the cuvette and start of the fluorescence measurement. Glucose oxidase (30 units/ml) was added 15 s before onset of actinic illumination. H_2O_2-additions to final concentrations of 4×10^{-4} M. KCN-concentration, 2×10^{-4} M. Actinic light intensity, 75 W/m^2. Temperature, 12 °C.

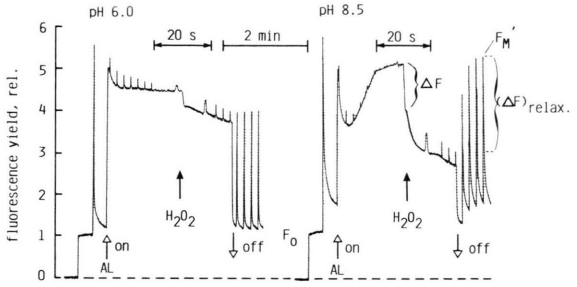

Fig. 5. H_2O_2-induced fluorescence quenching at pH 6.0 and 8.5 in the absence of molecular oxygen. Note: the recorder speed was increased fourfold for the indicated 20 s time spans in order to resolve the rapid component of H_2O_2-induced quenching (ΔF) starting at the F-level. The increase of F_M' after light-off is denoted (ΔF) relax. Conditions as for Fig. 4.

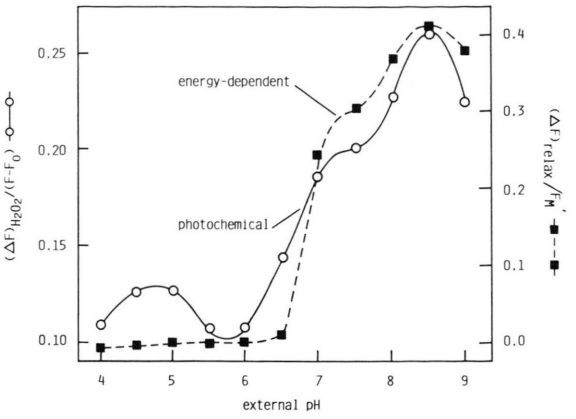

Fig. 6. Dependency of H_2O_2-induced fluorescence quenching on pH of external medium in the absence of molecular oxygen. H_2O_2-induced photochemical quenching is determined from $\Delta F(H_2O_2)$ normalized by $(F-F_0)$. H_2O_2-induced energy-dependent quenching is determined from the dark relaxation of F_M' within 40 s after light-off, (ΔF) relax., divided by F_M' determined 40 s after light-off. Conditions as for Figs. 4 and 5.

around pH 8.5. Both photochemical and energy-dependent quenching also display high values around pH 7, which may reflect the existence of two forms of ascorbate peroxidase with pH-optima close to 8.5 and 7. Alternatively, the optimum at pH 7 could be due to the ascorbate peroxidase [48] whereas the optimum at pH 8.5 could result from the MDA-reductase [48]. Only in photochemical quenching there is a minor peak close to pH 5. It also should be pointed out, that below pH 6.5 H_2O_2-induced energy-dependent quenching is practically zero, while there is still substantial H_2O_2-induced photochemical quenching. These data suggest that it is the peroxidase and MDA-reductase activities in the alkaline pH-range which provide the relevant electron flux for membrane energization.

The next question is: what is the pH-dependency of O_2-reduction? It should be possible to answer this question by measuring fluorescence quenching in presence of KCN which, as demonstrated in Fig. 4, inhibits H_2O_2-reduction. It is known that KCN also inhibits reactions involving the carboxylase/oxygenase. In Fig. 7 original kinetics traces of light-induced fluorescence changes in presence of KCN are compared for pH 5 and 8.5. Clearly, the photochemical quenching is substantially higher at pH 5 than at pH 8.5. And, most importantly, neither at pH 5 nor at pH 8.5 there is energy-dependent quenching developed, which would relax upon darkening. It appears that development of energy-dependent quenching depends on H_2O_2-reduction, and, as shown above, particularly on that part of H_2O_2-reduction which is catalyzed in the alkaline pH-range. In Fig. 8 the full pH-depend-

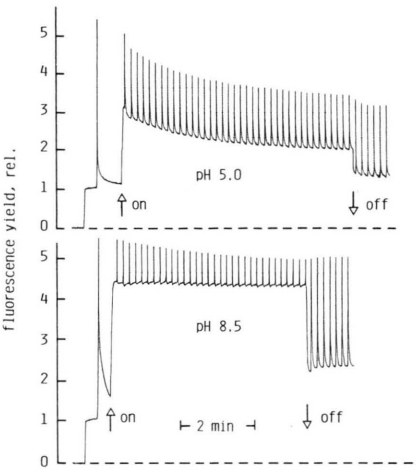

Fig. 7. Fluorescence induction kinetics of intact spinach chloroplasts suspended at pH 5.0 and 8.5. Assimilatory electron flow and H_2O_2-reduction were inhibited by 1 mM KCN. Light intensity, 20 W/m^2. Temperature, 20 °C. The observed photochemical quenching depends on O_2-reduction, as it disappears upon O_2-removal (not shown, but see Fig. 5).

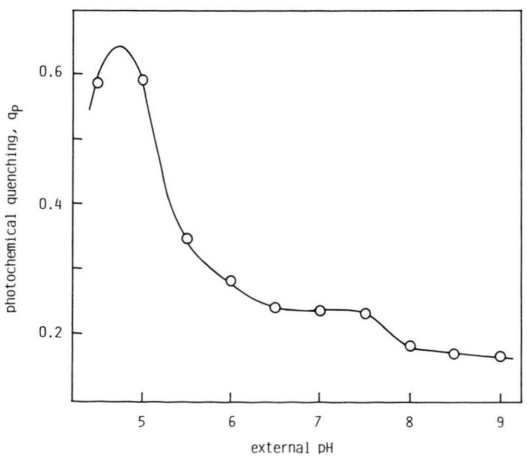

Fig. 8. Dependency on the external pH of the photochemical fluorescence quenching supported by O_2-reduction. Assimilatory electron flow and H_2O_2-reduction were inhibited by 1 mM KCN. Conditions as for Fig. 7.

ency of photochemical quenching caused by O_2-reduction is presented. Activity is relatively low in the alkaline pH-range and peaking close to pH 5.

It should be pointed out again, that the indicated pH-values are those of the external medium and do not necessarily correspond to the actual pH at the site of O_2-reduction. Nevertheless, the salient point is that O_2-reduction displays a pH-optimum in the acidic range contrasting with the major pH-optimum of H_2O_2-reduction in the alkaline range. Considering the obvious function of ascorbate peroxidase and MDA-reductase to reduce the H_2O_2 formed consequently to O_2-reduction, such contrasting pH-optima are surprising. However, the apparent discrepancy could be resolved, if O_2-reduction and H_2O_2-reduction would occur in different compartments of the chloroplast. Although there is a prevailing notion of O_2 being reduced by ferredoxin in the stroma [34, 43], there are also reports which point to a reduction of O_2 within the membrane phase [42, 51, 52]. In view of the surprising results of the present study, it appears that the *in vivo* role of intra-membrane O_2-reduction merits thorough investigation.

It may be asked whether the fluorescence quenching stimulated at low pH really reflects O_2-reduction. In principle, other acceptors (like nitrite) could also play a role and there is also the possibility of cyclic flow around PS II, which possibly is stimulated by low pH [53]. First, removal of O_2 (*e.g.* as in Fig. 4 and 5) completely suppresses photochemical quenching. Second, a stimulation of O_2-reduction by pH-lowering also is observed when O_2-uptake is measured polarographically with chloroplasts or photoacoustically with intact leaves (data not presented here). Briefly, it shall be mentioned that O_2-uptake is dramatically stimulated at pH 5–6, in particular when the ascorbate peroxidase is inhibited by KCN (Schreiber, Endo, Asada, and Neubauer, in preparation). And in intact tobacco leaves a strong uptake signal is revealed by pulse-modulated photoacoustic measurements [54], when the chloroplasts are acidified by application of high CO_2-concentrations (Reising and Schreiber, in preparation).

Conclusions

Fluorescence quenching analysis by the saturation pulse method has revealed that O_2-reduction and H_2O_2-reduction in intact chloroplasts display largely contrasting pH-optima close to pH 5 and 8.5 (external medium), respectively. Although there is some H_2O_2-induced photochemical quenching also in the pH 4–6 range, energy-dependent quenching is produced only by H_2O_2-reduction in the pH-range above 6.5. O_2-reduction does not lead to substantial energy-dependent quenching, neither at acidic nor at alkaline pH. With chloroplasts, which are suspended at pH 6–7, there is an increase of photochemical quenching during the first minute of illumination. As this increase is suppressed by uncouplers, it is proposed that the apparent activation results from the stimulation of H_2O_2-reduction upon light-induced alkalization of the stroma. And, as some of this activation is even observed in presence of KCN, it appears that also O_2-reduction is stimulated by ΔpH-formation. Such stimulation, however, would only agree with the observed acidic pH-optimum, if O_2-reduction would take place at the lumen side of the thylakoid membrane or within sequestered intra-membrane domains which are acidified by protolytic electron transfer reactions [55].

The presented results are important with respect to the regulation of photosynthetic electron flow. The transthylakoidal ΔpH and membrane energization reflected by energy-dependent quenching are known to have a decisive influence on the down-regulation of PS II when Calvin cycle activity becomes limiting in the presence of excess light. Our data suggest that it is the H_2O_2-reduction, being stimulated upon stroma alkalization, which results in the relevant membrane energization. If this suggestion is correct, the ascorbate peroxidase/MDA-reductase system provides not only, as so far assumed, an important detoxification mechanism, but also plays a decisive role in the regulation of *in vivo* electron transport. For this role a relatively low rate of H_2O_2-formation and reduction will be sufficient. It appears reasonable to assume that O_2-reduction and H_2O_2-formation will prevail only when NADPH can not be re-oxidized by the Calvin cycle. This situation may arise from a lack of ATP or from intrinsic limitations, *e.g.* insufficient CO_2-supply or stress-induced damage. As the reduction of O_2 and H_2O_2 does not require ATP, this "non-assimilatory" flux will be effective in producing excess ΔpH and membrane energization, which

then will cause a lowering of the intrinsic quantum yield of PS II, relieving the electron pressure on the PS II acceptor side and thus helping to prevent photoinhibitory damage. Important aspects inherent in this reaction sequence are: (1) The ΔpH which is formed with O_2-dependent electron flow will act stimulating on this very flow, *i.e.* there is mutually positive feedback, leading to autocatalytic behaviour. (2) The same ΔpH, which is known to slow down NADP-reduction by control of electron transfer from PQH_2 to Cyt *b/f*, will stimulate O_2-dependent flow. The resulting model of the regulatory role of O_2-dependent flow is depicted schematically in Fig. 9.

Finally, it may be concluded that the apparent activation of O_2-dependent electron flow could be the basis of the well-known Kautsky effect of chlorophyll fluorescence induction [56, 57]. It has been known for long that with dark-adapted samples O_2 and ΔpH-formation are required for the decline of fluorescence from the initial peak to the steady state level [57–59]. We have suggested earlier that it is "a reactant which is formed as a consequence of O_2-reduction" which is involved [12]. It now appears that it is the H_2O_2, the ΔpH and the proposed autocatalytic reaction sequence which lead to activation of primary electron flow. Furthermore, it appears that the "regulatory oscillations" of various photosynthetic parameters which are observed upon sudden changes of light intensity, CO_2- or O_2-concentration [60–62] may also be related to the regulatory mechanisms associated with O_2-dependent electron flow. More experimentation will be required to obtain final evidence for these suggestions.

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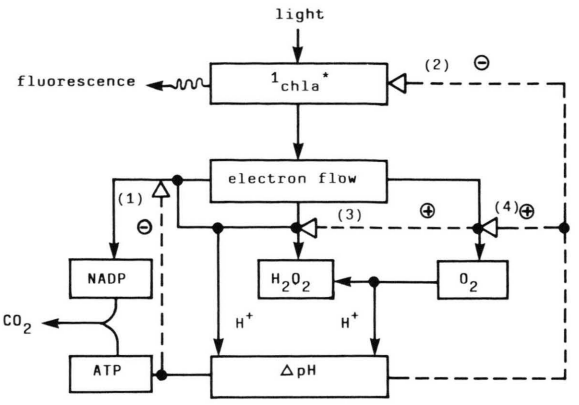

Fig. 9. Regulation of photosynthesis by O_2-dependent electron flow. Assimilatory electron flow is limited by the stoichiometric demands of ATP/NADPH, with at least as much H^+ consumed for ATP-synthesis as translocated to form ΔpH. Maintenance of ΔpH primarily depends on O_2-dependent flow, involving O_2- and H_2O_2-reduction. It is suggested that the ΔpH exerts regulatory control at 4 sites (indicated by open arrows, with ⊕ and ⊖ denoting positive and negative feedback, respectively): (1) Slow-down of NADP-reduction (control at $PQH_2 \rightarrow$ Cyt *b/f*). (2) Down-regulation of PS II intrinsic quantum yield (dissipation of singlet excitation energy). (3) Stimulation of H_2O_2-reduction (by stroma alkalization). (4) Stimulation of O_2-reduction (by membrane acidification?). Different time constants for the 4 different ΔpH-effects may be assumed, with feedback reaction (1) presumably being as fast as ΔpH-formation; reaction (2) depends on slow conformational changes paralleling the development of energy-dependent fluorescence quenching; reaction (3) is slowed down by the buffer capacity of the stroma; reaction (4), if indeed intra-membraneous, could be as fast as reaction (1), *i.e.* the internal acidification which slows down NADP-reduction may cause an equivalent stimulation of O_2-reduction. O_2-reduction and consequent H_2O_2-reduction will lead to extra-ATP production which again will stimulate assimilatory flow and slow down O_2-dependent flow. If CO_2-assimilation is intrinsically limited, *e.g.* by CO_2-supply or stress-induced damage, O_2-dependent flow will prevail, leading to sustained down regulation of PS II.

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